Exhibit C

Protein microarray technology

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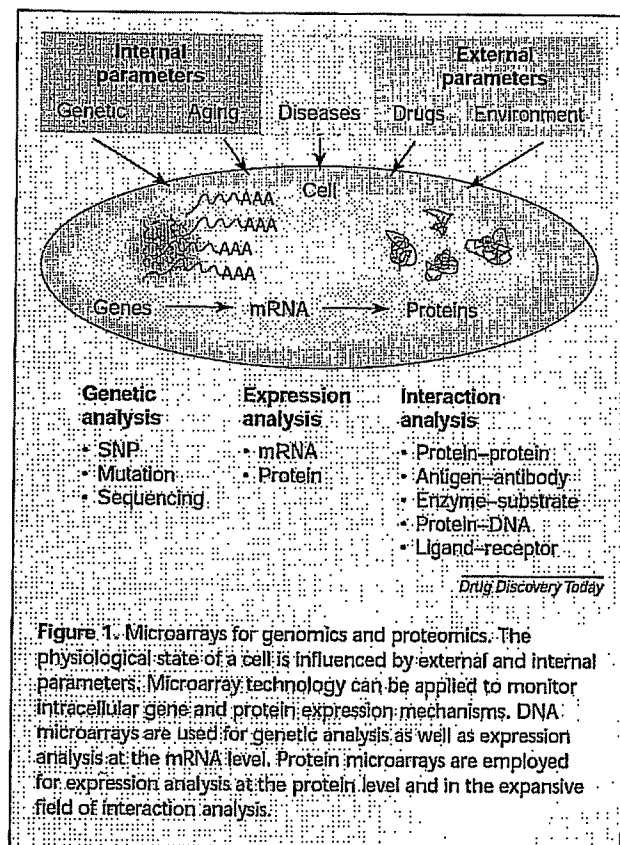
Microarray technology allows the simultaneous analysis of thousands of parameters within a single experiment. Microspots of capture molecules are immobilised in rows and columns onto a solid support and exposed to samples containing the corresponding binding molecules. Readout systems based on fluorescence, chemiluminescence, mass spectrometry, radioactivity or electrochemistry can be used to detect complex formation within each microspot. Such miniaturised and parallelised binding assays can be highly sensitive, and the extraordinary power of the method is exemplified by array-based gene expression analysis. In these systems, arrays containing immobilised DNA probes are exposed to complementary targets and the degree of hybridisation is measured. Recent developments in the field of protein microarrays show applications for enzyme–substrate, DNA–protein and different types of protein–protein interactions. This article discusses theoretical advantages and limitations of any miniaturised capture-molecule–ligand assay system and discusses how the use of protein microarrays will change diagnostic methods and genome and proteome research.

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The fundamental principles of miniaturised and parallelised microspot ligand-binding assays were described more than a decade ago. In the 'ambient analyte theory', Roger Ekins and coworkers [1–4] explained why microspot assays are more sensitive than any other ligand-binding assay. At that time, the high sensitivity and enormous potential of microspot technology had already been demonstrated using miniaturised immunological assay systems. Nevertheless, the enormous interest that microarray-based assays evoked came from work using DNA chips. The possibility of determining thousands of different binding events in one reaction in a massively parallel fashion perfectly suited the needs of genomic approaches in biology. The rapid progress in whole-genome sequencing (e.g. [5,6]) and the increasing importance of expression studies (expressed sequence tag [EST] sequencing) was matched with efficient in vitro techniques

for synthesising specific capture molecules for ligand-binding assays. Oligonucleotide synthesis and PCR amplification allow thousands of highly specific capture molecules to be generated efficiently. New trends in technology, mainly in microtechnology and microfluidics, newly established detection systems and improvements in computer technology and bioinformatics were rapidly integrated into the development of microarray-based assay systems. Now, DNA microarrays, some of them built from tens of thousands of different oligonucleotide probes per square centimetre, are well-established high-throughput hybridisation systems that generate huge sets of genomic data within a single experiment (Fig. 1). Their use for the analysis of single nucleotide polymorphisms and in expression profiling has already changed pharmaceutical research, and their use as diagnostic tools will have a big impact on medical and biological research.
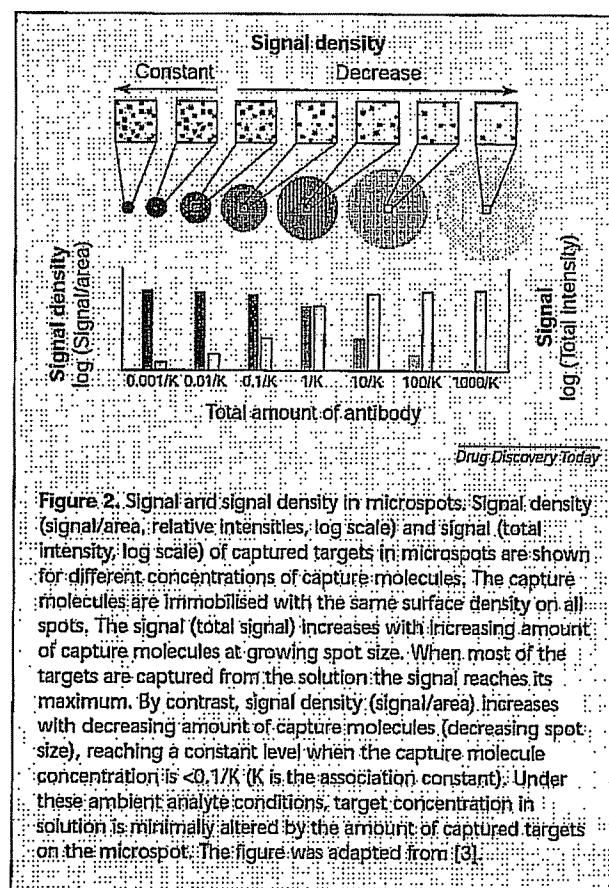
As known from gene expression studies, however, mRNA level and protein expression do not necessarily correlate [7–9]. Protein functionality is often dependent on post-translational processing of the precursor protein and regulation of cellular pathways frequently occurs by specific interaction between proteins and/or by reversible covalent modifications such as phosphorylation. To obtain detailed information about a complex biological system, information on the state of many proteins is required. The analysis of the proteome of a cell (i.e. the quantification of all proteins and the determination of their post-translational modifications and how these are dependent on cell-state and environmental influences) is not possible without novel experimental approaches. High-throughput protein analysis methods allowing a fast, direct and quantitative detection are needed. Efforts are underway, therefore, to expand microarray technology beyond DNA chips and
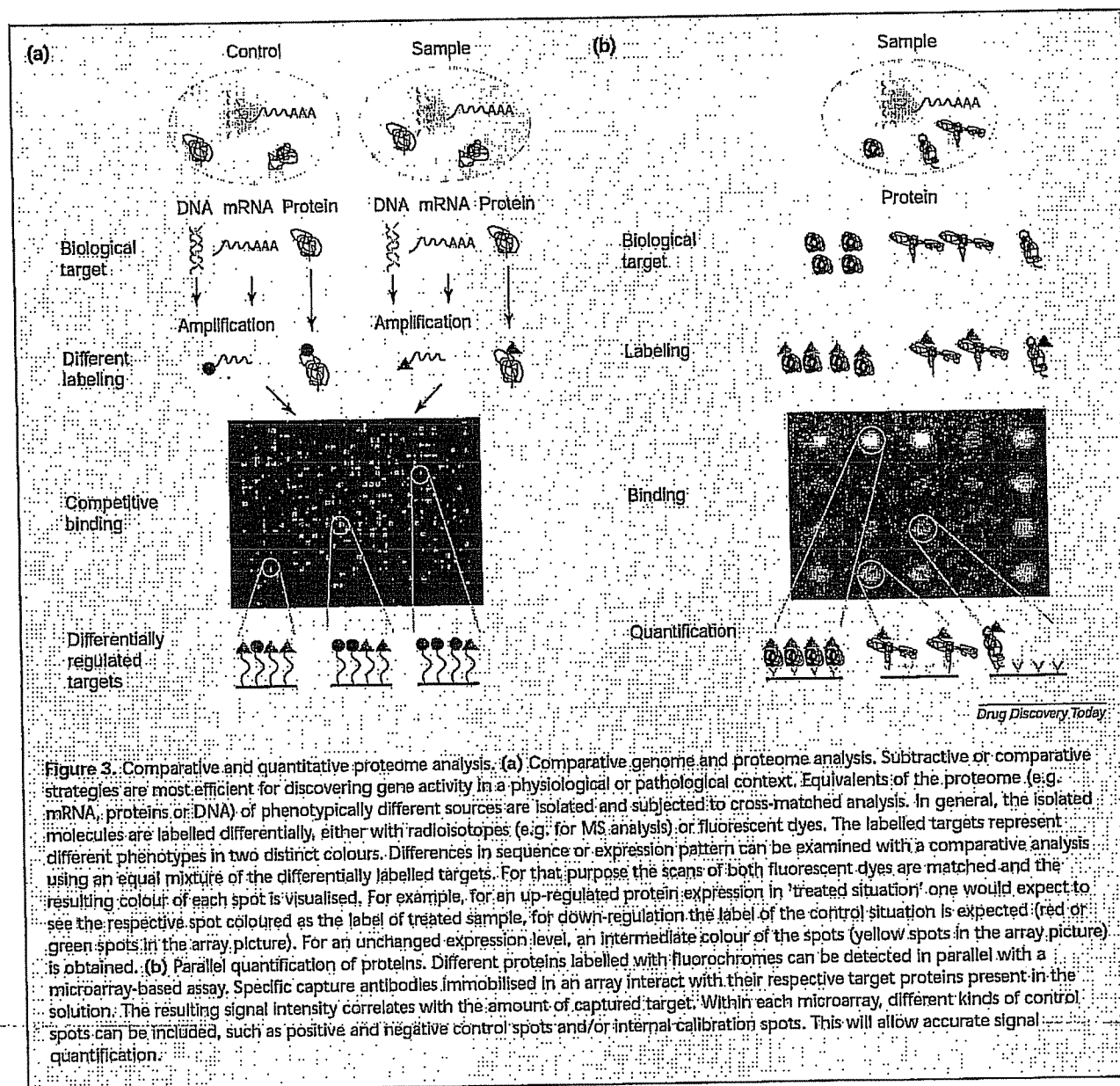
Figure 1. Microarrays for genomics and proteomics. The physiological state of a cell is influenced by external and internal parameters. Microarray technology can be applied to monitor intracellular gene and protein expression mechanisms. DNA microarrays are used for genetic analysis as well as expression analysis at the mRNA level. Protein microarrays are employed for expression analysis at the protein level and in the expansive field of interaction analysis.

Figure 2. Signal and signal density in microspots. Signal density (signal/area, relative intensities, log scale) and signal (total intensity, log scale) of captured targets in microspots are shown for different concentrations of capture molecules. The capture molecules are immobilised with the same surface density on all spots. The signal (total signal) increases with increasing amount of capture molecules at growing spot size. When most of the targets are captured from the solution the signal reaches its maximum. By contrast, signal density (signal/area) increases with decreasing amount of capture molecules (decreasing spot size), reaching a constant level when the capture molecule concentration is <0.1/K (K is the association constant). Under these ambient analyte conditions, target concentration in solution is minimally altered by the amount of captured targets on the microspot. The figure was adapted from [3].

establish array-based approaches to characterise proteomes (Fig. 1) [10–12].

Miniaturised ligand-binding assays: theoretical considerations

The ambient analyte assay theory shows that miniaturised ligand-binding assays are able to achieve a superior sensitivity. A system that uses a small amount of capture molecules and a small amount of sample can be more sensitive than a system that uses a hundred times more material. Ekins and coworkers [1–4] developed a sensitive microarray-based analytical technology and proved the high sensitivity of the miniaturised assay. With this system, analytes, such as thyroid stimulating hormone (TSH) or Hepatitis B surface antigen (HbsAG), could be quantified down to the femtomolar concentration range (corresponding to 10^6 molecules ml^{-1}). Miniaturisation is the key to understanding the principle of miniaturised binding assays. Capture molecules are immobilised to the solid phase only in a very small area, the microspot – although the amount of capture molecules present in the system is low, a high density of molecules in the microspot can be obtained (Fig. 2). During an assay, target molecules, or analytes, are captured by the microspot but the number of

capture–target complexes is low owing to the small area of the microspot. As a result, the capture process does not change the concentration of the target molecules in the sample significantly, even for targets present in low concentration and for binding reactions that occur with a high affinity. This is true if <0.1/K of capture molecules get immobilised, where K is the affinity constant of the binding reaction. These conditions, termed ambient analyte assay, allow measurements where the amount of the target or analyte captured from solution directly reflects its concentration in the assay system. Interestingly, the concentration measurement under ambient analyte conditions makes the system independent of the actual volume of sample used and it can give results that combine high sensitivity with low sample consumption. The sensitivity that can be obtained is high for two reasons. First, the binding reaction occurs at the highest possible target concentration. Second, the capture-molecule–target complex is found only in the small area of the microspot, resulting in a high signal (Fig. 2). Capture molecules are immobilised in a constant surface density onto spots that have an increasing spot size. With increasing spot size, the total amount of capture molecules present in an assay

Figure 3. Comparative and quantitative proteome analysis. (a) Comparative genome and proteome analysis. Subtractive or comparative strategies are most efficient for discovering gene activity in a physiological or pathological context. Equivalents of the proteome (e.g. mRNA, proteins or DNA) of phenotypically different sources are isolated and subjected to cross-matched analysis. In general, the isolated molecules are labelled differentially, either with radioisotopes (e.g. for MS analysis) or fluorescent dyes. The labelled targets represent different phenotypes in two distinct colours. Differences in sequence or expression pattern can be examined with a comparative analysis using an equal mixture of the differentially labelled targets. For that purpose the scans of both fluorescent dyes are matched and the resulting colour of each spot is visualised. For example, for an up-regulated protein expression in 'treated situation' one would expect to see the respective spot coloured as the label of treated sample, for down-regulation the label of the control situation is expected (red or green spots in the array picture). For an unchanged expression level, an intermediate colour of the spots (yellow spots in the array picture) is obtained. (b) Parallel quantification of proteins. Different proteins labelled with fluorochromes can be detected in parallel with a microarray-based assay. Specific capture antibodies immobilised in an array interact with their respective target proteins present in the solution. The resulting signal intensity correlates with the amount of captured target. Within each microarray, different kinds of control spots can be included, such as positive and negative control spots and/or internal calibration spots. This will allow accurate signal quantification.

increases, as does the sum signal obtained from the spot. The signal density, however, starts to decrease with increasing spot size because the amount of target starts to become a limiting factor. The capture process leads to a significant reduction of target concentration in solution and at the same time the probe–target complexes get distributed over a larger area. As a result, the maximal signal that can be obtained from any point in a spot is decreased. Decreasing the spot size will decrease the overall signal per microspot but the signal density will increase for smaller spots (Fig. 2). Below a certain spot size, the signal-density approaches an optimum (ambient analyte conditions; amount of target not limiting) and will stay approximately constant with

any further decrease in spot size. Therefore, the highest signal intensities and optimal signal-to-noise ratios can be achieved in small spots.

Microarray technology

For DNA microarrays, presynthesized oligonucleotides or PCR-fragments are immobilised because capture molecules or oligonucleotides can be synthesised directly on a solid support [13,14]. Targets to be analysed are extracted from the cell, labelled and hybridised to their immobilised complementary capture probes (Fig. 3) and captured targets are measured and quantified. DNA-chip technology takes advantage of the fact that a large number of targets can be

Table 1. Classes of capture molecules

Capture molecules	Source	Technique	Refs
mAb	Mouse	Hybridoma	Reviewed in [42,43]
scFv/Fab diabodies	Antibody libraries	Phage display; *in vitro* evolution	[16–19,44–47]
Affinity binding agents	Recombinant fibronectin structures	*In vitro* evolution	[48]
Affibodies	Microorganism	Heterologous expression	[49,50]
Aptamers (DNA/RNA/peptide)	Library	SELEX/mRNA display *in vitro* evolution	[23,24,51–54]
Receptor ligands	Synthetic	Combinatorial chemistry	[36,55]
Substrates of enzymes	Synthetic; pro- and eukaryotic organisms	Protein purification: Recombinant protein technology (bacterial fusion proteins; baculovirus; peptide synthesis)	[33,34,37]

Abbreviations: fab, antigen-binding fragment; mAb, monoclonal antibody; scFv, single-chain variable region fragment. This table summarises classes of molecules that have the potential to be used or are actually used as capture molecules in protein microarray systems. The sources they are from and the key technologies used to generate such capture molecules are listed together with selected relevant references.

analysed in massive parallel measurements with low sample consumption. Furthermore, it is possible to perform comparative analyses of two different samples with a single array. The targets in the two samples are labelled with two different fluorophores, equal sample amounts are mixed and hybridised simultaneously to the same microarray. The ratio of the two differentially labelled target signals on each microspot directly reveals whether the targets are present in different or similar concentrations (Fig. 3).

As a direct transfer from the DNA chip technology, Haab *et al.* [15] adapted the same dual colour labelling procedure to antibody–antigen microarrays (Fig. 3). They used a set of 110 defined antigen–antibody pairs to create microarray-based immunoassays as a proof of concept for differential protein analysis. Either antibodies or antigens were immobilised and the corresponding targets were fluorescently labelled in complex solutions such as serum. One sample spiked with antigens was labelled with one fluorophore and another sample containing a different concentration of the antigens was labelled with a different fluorophore. The two samples were mixed and incubated simultaneously on the same microarray. A dual colour detection system immediately revealed the different concentrations of the captured targets. Depending on the affinity of the antibody, picomolar concentrations of antigens were detectable. However, proteins are often assembled as complexes and as a result, a strong signal on a microspot can result from a large amount of target or from the capture of a huge complex. In general, their results demonstrated that the comparative analysis approach can, in principle, be transferred from DNA to proteins. When switching from DNA to proteins, one has to be aware that DNA and proteins are

different classes of molecules with different chemical and physical properties. DNA is built out of four different nucleotides, which generate a uniform molecule with a well-defined structure and a hydrophilic, negatively charged sugar backbone. By contrast, proteins are made from 20 different amino acids resulting in highly diverse molecules with different abilities. Proteins can be hydrophilic or hydrophobic, acidic or basic and post-translationally modified (glycosylation, acetylation or phorphorylation). In addition, proteins have diverse and individual molecular structures. Furthermore, the capture proteins must be kept in a functional state, when immobilised onto a microarray.

Antibodies are the most prominent capture molecules used to identify targets. Owing to the labour intensive nature of monoclonal antibody production, however, the development of other alternatives has become crucial. The most promising approaches in this field are phage-display techniques [16,17] combined with highly diverse (up to 10^{11} independent clones), fully synthetic libraries to generate artificial antibodies [18,19]. Another strategy is the generation of highly specific oligonucleotide [20–22] or peptide aptamers (Table 1, Fig. 4). The production and selection (e.g. SELEX; see [23]) of these molecules are amenable to automation and high-throughput capture molecule generation. mRNA display methodology [24] allows the quick and efficient production of ultra diverse libraries, leading to binder molecules directed to almost any target with nM–pM affinities.

The growing field of protein microarray technology also requires the development of methods for high-throughput generation of recombinant proteins. Such methods are a prerequisite for the growing demand for thousands of

specific capture molecules. Only the successful solution of this challenge will enable progression to high-density protein microarrays, which will be key tools for any array-based proteomic approach [25].

Enormous efforts are underway to automate recombinant protein expression and purification. Miniaturised expression systems for the massive parallel generation of recombinant proteins in *Escherichia coli* and in the baculovirus system have already been developed [10,11]. High-density fusion protein microarrays have already been generated and used for antibody-specificity screening [26,27], as well as high-density single-chain antibody library arrays for the identification of appropriate antigens [28].

In addition to their use in the generation and isolation of appropriate capture molecules, recombinant proteins will be used to generate microarrays that allow a rapid efficient screening for high-affinity binders with minimal or no cross-reactivity to other proteins. Selectivity of the capture molecules will be the most crucial issue in the context of all array-based proteomic approaches.

Meanwhile, the products necessary for the creation and for the performance of a DNA microarray experiment are commercially available. There are more than a hundred companies on the market who offer products for the DNA microarray community (see http://www.biochipnet.de). These technical devices can be also used directly or after slight modifications for the generation of protein arrays. Most supports used for the immobilisation of microarrays are made from glass but plastics and polymer membranes can also be used. The immobilisation of proteins is often done using noncovalent protein surface interactions with hydrophobic (nitrocellulose, polystyrene) or positively charged (poly-lysine, aminosilane) surfaces. Covalent attachment using a variety chemically activated surfaces (e.g. aldehyde, epoxy, active esters) is used as well as attachment by specific bimolecular interactions (e.g. streptavidine-biotin, His-tag-nickel-chelates) for the generation of protein microarrays. The production of tiny microspots on such surfaces is performed using contact printing arrayers with tiny needles placing sub-nanolitre sample volumes directly on the surface. Alternatively, non-contact deposition technologies are used that apply capillaries or ink jet technology to deposit nanolitre–picolitre droplets onto the surface.

Detection of captured targets is mainly performed by fluorescence using charge-coupled device (CCD) cameras or laser scanners with confocal detection optics. Furthermore, radioactivity, chemiluminescense or label-free plasmon-resonance-based detection systems can also be used. The surface-enhanced laser desorption and ionisation (SELDI) technology adapted mass spectrometry as a read-out system
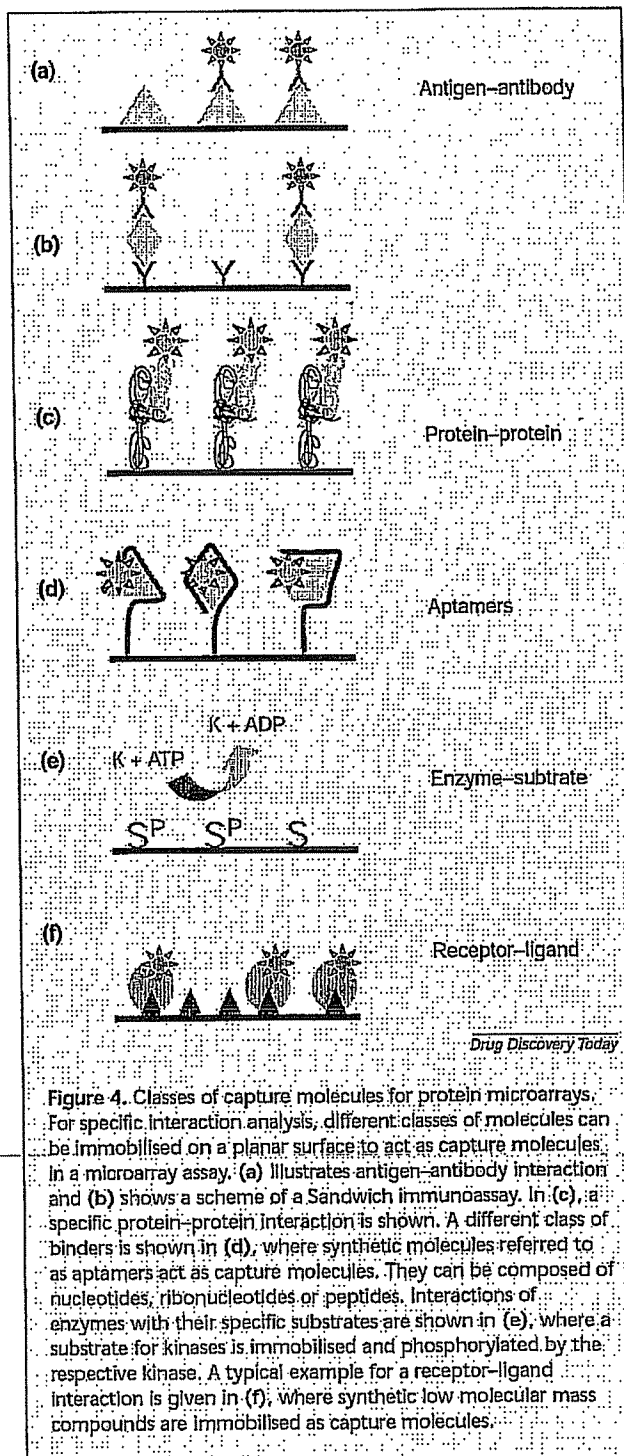

Figure 4. Classes of capture molecules for protein microarrays. For specific interaction analysis, different classes of molecules can be immobilised on a planar surface to act as capture molecules in a microarray assay. (a) illustrates antigen–antibody interaction and (b) shows a scheme of a Sandwich immunoassay. In (c), a specific protein–protein interaction is shown. A different class of binders is shown in (d), where synthetic molecules referred to as aptamers act as capture molecules. They can be composed of nucleotides, ribonucleotides or peptides. Interactions of enzymes with their specific substrates are shown in (e), where a substrate for kinases is immobilised and phosphorylated by the respective kinase. A typical example for a receptor–ligand interaction is given in (f), where synthetic low molecular mass compounds are immobilised as capture molecules.

to analyse differential protein expression on spot arrays [29]. Cell extracts derived from different origins are incubated on different spots of the same adsorptive surface chemistry (e.g. cation–anion exchange material, hydrophobic surface). After washing away unbound proteins, the

whole variety of non-specifically captured target proteins can be analysed using SELDI mass spectroscopy. The mass spectrum shows the different molecular weights of the captured proteins. Comparison of two mass spectroscopy data sets generated from two different samples immediately identifies the differentially expressed proteins. In some cases, the differentially displayed proteins can be identified immediately by their molecular weights but usually these proteins have to be enriched by affinity chromatography and identified by methods known from protein analysis (such as Edman sequencing, western blot and digest mass fingerprinting) [30].

Protein microarrays

In principle, any kind of ligand-binding assay that relies on the product formation of an immobilised capture molecule and a target (binder or analyte) present in the surrounding solution can be miniaturised, parallelised and performed in a microarray format. Microarray-based assays using nucleic acid–nucleic acid interactions (DNA chips) are well established and protein microarray assays are just becoming popular. This can be seen by recent publications about microarray-based assays discussing nucleic acid–protein, protein–protein, ligand–receptor and enzyme–substrate interactions (Fig. 4) [31–37].

Studies on DNA–protein interactions in a microarray format were performed by Bulyk et al. [32] who created microarrays of double-stranded oligonucleotides. High-density microarrays of single-stranded oligonucleotides were produced using Affymetrix (Santa Clara, CA, USA) technology. This single-stranded oligonucleotide microarray was converted into a double-stranded oligonucleotide microarray by an enzymatic extension reaction. These arrays were incubated with a restriction enzyme specific for distinct DNA sequences. No DNA-cleavage occurred when the double-stranded DNA was enzymatically methylated before incubation with the restriction enzyme. In general, DNA–protein interaction assays could be useful for the characterisation and identification of DNA-binding proteins, such as transcription factors.

Enzyme–substrate arrays have been described for different kinds of enzymes, such as restriction enzymes, peroxidase, phosphatase and protein kinases [32–34,37]. In a proof of concept experiment, MacBeath and Schreiber [37] immobilised three different kinase substrates, each specific for an individual kinase, onto a planar glass surface. Identical microarrays were incubated each with one individual kinase together with radioactively labelled ATP. Each substrate was only phosphorylated by its specific kinase. In a more advanced approach, Zhu et al. [34] analysed the activity of 119 different protein kinases from Saccharomyces cerevisiae for 17 different substrates. They used microwell plates in which substrates were covalently attached to individual microwells. The kinases expressed as glutathione-S-transferase (GST) fusion proteins were incubated with their substrates together with radioactively labelled ATP in each microwell. After finishing the kinase reaction, the kinases and the ATP were washed away and the arrays were analysed for phosphorylated substrates with a phosphoimager. Using this approach, novel activities of individual kinases were identified. Sequence comparison of enzymes that could phosphorylate tyrosine residues revealed that they often share common amino acid residues around their catalytic region.

For receptor–ligand assays, small organic molecules produced by combinatorial solid phase chemistry were immobilised in a microarray format. Single resin beads from combinatorial synthesis were placed in 96-well plates and the organic molecules were chemically released from the beads. The organic molecules were diluted, spotted and covalently attached on derivatised glass slides. These microarrays produced by so-called small-molecule printing technology were incubated with fluorescently labelled target proteins to identify new ligands [36]. This technology enables parallel high-throughput screening for ligand–receptor interactions at very low sample consumption, which could improve screening for active substances in the pharmaceutical industry.

In the field of protein–protein interaction assays, dot-blot filter arrays were used to screen for specific interactions of immobilised proteins with other proteins. Specific protein–protein interactions were detected between a radioactively labelled human p52 GST fusion protein and immobilised capture proteins such as nucleoline or a serine-argenine protein fraction isolated from HeLa cells [35]. In addition, interactions of DNA, RNA, or low molecular weight ligands with the immobilised molecules were shown. Such arrays could be further miniaturised and therefore have the potential to be performed in a microarray format.

Recent work by Zhu et al. [38] demonstrated the extraordinary power of array-based methods for proteomic approaches. After purification of 5800 different recombinant proteins from S. cervisiae, the authors generated complex proteome chips that contained gene-products from >90% of the genes of the organism. These microarrays could be used to study protein–protein interactions on a genome-wide scale. Using calmodulin as a model protein to probe the arrays, many known interactions could be confirmed and a set of novel binding proteins was detected. Inspection of the sequences of these proteins revealed the presence a binding motif and therefore strongly argues for

the significance of the observed binding interaction. Experiments designed to detect protein–lipid interactions convincingly showed that the identification of proteins able to bind low molecular weight compounds is possible. This opens the possibility to examine an entire proteome directly for protein–drug interactions.

Microarray immunoassays are of general interest for all diagnostic applications where several parameters of one sample have to be analysed in parallel [31,39,40]. We used microarray technology to screen for antigen–antibody interactions. Eighteen different autoantigens, commonly used as diagnostic markers for autoimmune diseases such as systemic rheumatic diseases, were immobilised in a microarray allowing the parallel detection of the different types of autoantibodies. From <1 microlitre of patient serum autoantibody titers were determined with high accuracy [31]. Sandwich immunoassays were also miniaturised and parallelised and performed in a microarray format. This was recently demonstrated by the parallel determination of different cytokine levels in biological samples with high specificity and sensitivity [41].

Accurate quantification with protein microarrays can be achieved by including positive and negative control spots and/or internal calibration spots (Fig. 3b). This will finally lead to robust and reliable diagnostic assays.

Conclusions

The examples given above show that protein microarray technology is already a useful tool to study different kinds of protein interactions. Further developments and optimisation of array production and assay performance combined with high-throughput generation of protein targets and ligands will extend the number of applications of protein microarrays dramatically. Proteomic research and diagnostic applications will be the two major fields addressed by protein microarray technologies.

In medical research, protein microarrays will accelerate immune diagnostics significantly by analysing in parallel all relevant diagnostic parameters of interest. The reduction of sample volume is of great importance for all applications in which only minimal amounts of samples are available. One example might be the analysis of multiple tumour markers from a minimum amount of biopsy material. Furthermore, new possibilities for patient monitoring during disease treatment and therapy will be developed based on this emerging technology.

Microarray-based technology beyond DNA chips will accelerate basic research in the area of protein–protein interactions and will allow protein profiling from limited numbers of proteins up to high density array-based proteomic approaches. Protein and peptide arrays will be used to analyse enzyme–substrate specificity and for measurement of enzyme activity on different kinds of substrates in a highly parallel fashion.

The whole field of protein microarray technology shows a dynamic development driven by the increasing genomic information. New technologies such as automated protein expression and purification systems, used for the generation of capture molecules and the need for analysis of whole 'proteomes' will be a driving force for fast developments within the field of protein microarray technology. The story is just beginning!

Acknowledgements

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